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Physical characteristics of the genome of the phytopathogenic fungus *Puccinia graminis*

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Abstract. The physical characteristics of the genome of *Puccinia graminis* f. sp. *tritici*, the wheat stem rust fungus, were determined by reassociation kinetics. The results indicate that the haploid genome contains 67 Mb and consists of three classes of DNA sequences: (1) 64% unique; (2) 30% repetitive; and (3) 4% foldback. The repetitive sequences have a total complexity of 390 kb and are repeated an average of 52 times. The base composition was 45.3% G + C based on an analysis of the DNA melting temperature. The average amount of DNA per ungerminated urediniospore by diphenylamine assay, corrected for losses during extraction, was 435 fg. This was three times the expected value (147 fg) for dikaryotic spores with nuclei in the G_1 phase of the cell cycle, an indication that the spores were in G_2.

Key words: *Puccinia graminis* – Genome size – Reassociation kinetics – Repetitive DNA

Introduction

The physical characteristics of a variety of fungal genomes have been investigated by analysis of DNA reassociation kinetics (Firtel and Bonner 1972; Hudspeth et al. 1977; Krumlauf and Marzluf 1979; Ullrich et al. 1980; Arthur et al. 1982; Wöstemeyer and Burmester 1986). Fungal genomes span a considerable range in size, from 13.6 Mb for *Saccharomyces cereviseae* (Lauer et al. 1977) to 62 Mb for *Phytophthora megasperma* f. sp. *glycinea* (Mao and Tyler 1991). In addition to providing an estimate of the overall size, analysis of DNA reassociation kinetics provides information on the nature of the repetitive sequences contained within the genome. The repetitive DNA content of fungi has been shown to vary over a broad range, from essentially no repetitive DNA in *S. cereviseae* (Lauer et al. 1977) and *Aspergillus niger* (Timberlake 1978) to approximately 60% in *Bremia lactucae* (Francis et al. 1990). At least 20 fungal genomes have been characterized by reassociation kinetics analysis (Mao and Tyler 1991).

Despite their prominence as plant pathogens, little is known about the genome size and repetitive DNA content of the cereal rust fungi. Rust pathogens cause serious economic losses in cereals. Furthermore, they are race-specific following a well-documented gene-for-gene relationship with cereal cultivars. As a model for cereal rust pathogens, we have chosen to study *Puccinia graminis* Pers. f. sp. *tritici*, the causal agent of wheat stem rust. Epidemics of wheat stem rust, potentially the most destructive of all wheat diseases, have periodically caused severe losses in many parts of the world (Roelfs 1985). *P. graminis* is a basidiomycetous, obligate parasitic fungus with a complex life cycle which includes five spore stages and two hosts. The asexual uredinial phase is the economically important part of the life cycle. Dikaryotic urediniospores produced on wheat can reinfect wheat and a number of other *Triticum* species in repeated disease cycles over the course of a growing season. The uredinial part of the life cycle is governed by race-specific resistance and currently more than 50 resistance/avirulence gene pairs have been characterized (Roelfs 1985). Whether development on the alternate host is governed by a similar gene-for-gene system is not known.

Toward the goal of describing host-parasite interactions in the cereal rusts at the molecular level, we have begun to characterize the genome of *P. graminis*. Here we report an estimate of the haploid genome size and the amount of repetitive DNA based on an analysis of DNA reassociation kinetics. Also, we have used chemical analysis to determine the total amount of DNA in ungerminated urediniospores. The results provide information on the physical nature of the genome needed for developing strategies for gene cloning and for the construction of genetic and physical maps.

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Materials and methods

Biological materials. Urediniospores from the isolate SZA-8D (CRL # 74-36-924-A, Race Pgt-SCM) of *P. graminis* f. sp. *tritici* were increased in the greenhouse (Rowell 1984) and stored at 9.5 °C and 30% relative humidity until used. The genetic homogeneity of the increased urediniospores was confirmed using standard avirulence differentials (Roelfs and Martens 1988). *Escherichia coli* strain W3110 (*E. coli* Genetic Stock Center #4474, kindly supplied by Dr. B. J. Bachmann) was used as a standard in reassociation kinetics experiments. This strain has been cloned in its entirety as a set of overlapping lambda clones and, based on the derived physical map, its genome size has been determined to be 4.7 Mb (Kohara et al. 1987).

DNA purification. P. graminis urediniospores were disrupted using an MSK Braun homogenizer (B. Braun Biotech Inc., Allentown, Pa.) essentially as described (Rick et al. 1966). Six grams of urediniospores were disrupted in a 75-ml MSK homogenizer flask containing 40 g of 0.5-mm glass beads and 20 ml of extraction buffer (200 mM Tris-HCl, pH 8.0; 250 mM NaCl; 100 mM EDTA). The spores were homogenized for 3 min at 4000 rpm with periodic cooling by CO_2. Following disruption, one-tenth volume of 10% SDS was added to the homogenate which was then extracted with an equal volume of chloroform:isoamyl alcohol (24:1, v:v) and subjected to equilibrium centrifugation in CsCl-ethidium bromide gradients (Sambrook et al. 1989). Additional DNA purification was achieved by means of hydroxyapatite (HAP) chromatography (Bernardi 1971). The DNA was precipitated, re-suspended in 0.12 M NP (an equimolar mixture of Na_2HPO_4 and NaH_2PO_4), and applied to a HAP column equilibrated in 0.12 M NP. The column was washed with the starting buffer until the A_{260} reached the baseline and the DNA was then eluted with a gradient formed from equal volumes of 0.12 M NP and 0.4 M NP. DNA in the peak fractions was recovered by sedimentation and re-suspended in TE buffer (10 mM Tris-HCl, pH 7.6; 1 mM EDTA). *E. coli* DNA was extracted and purified using essentially the same procedure. The cells from 1.5 l of late log-phase bacterial culture were disrupted in a 75-ml MSK homogenizer flask with 52 g of 0.25-mm glass beads and 20 ml of extraction buffer. Following HAP purification, the *E. coli* DNA was additionally purified by a second round of CsCl-ethidium bromide equilibrium density gradient centrifugation.

Determination of DNA melting temperature (T_m). Purified *P. graminis* DNA in 0.12 M NP was heated in a temperature-controlled cuvette assembly while the absorbance at 260 nm was monitored with a Beckman DU8 spectrophotometer. The temperature was raised from 45 °C to 102 °C in one degree intervals and, after each step, the samples were allowed to equilibrate for 1.5 min before the absorbance was determined. The T_m of *Clostridium perfringens* DNA (Sigma cat. # D 5139) in 0.12 NP was found to be 81.0 °C at this cation concentration (Marmur and Doty 1962; Cantor and Schimmel 1980).

Reassociation kinetics. The reassociation kinetics of *P. graminis* and *E. coli* DNA were characterized using standard methods (Britten et al. 1974). The tracer DNA was tritium labeled by nick translation (Durnam and Palmiter 1983) and the nicks sealed using T4 DNA ligase (Sambrook et al. 1989). Labeled DNA was size-fractionated by means of alkaline-gel electrophoresis (Sambrook et al. 1989) and fragments larger than 500 nt were recovered by electroelution. Labeled and unlabeled DNA were then combined in a ratio of 1:15000, brought to 66% glycerol, and sheared with a Sorvall Omni mixer operated at maximum speed for 30 min using a 10-ml vessel and a micro-attachment. The DNA was ethanol-precipitated, resuspended in TE buffer and passed over a Chelex (Bio-Rad Laboratories, Richmond, Calif.) column to remove divalent cations. The average single-stranded length of both labeled and unlabeled fragments of *P. graminis* DNA following this procedure was 420 nt as determined by alkaline agarose-gel electrophoresis. Aliquots containing 33 to 39 µg of DNA were prepared in 0.12 M NP at concentrations ranging from 0.040 to 3.61 mg/ml. Samples were flame-sealed in glass capillary tubes or, for the most dilute samples (0.040 mg/ml), placed in gas-tight microfuge tubes. To denature the DNA, the tubes were immersed in a boiling water bath for 1 min (capillary tubes) or 4 min (microfuge tubes). The tubes were then incubated at 60 °C for periods ranging from 30 s to 72 h. Immediately following the incubation period, the samples were brought to 1 ml with 0.12 M NP and fractionated on a water-jacketed column containing 1 ml of HAP. The columns, as well as all solutions, were maintained at 60 °C. Single-stranded DNA was eluted in five 1-ml washes of 0.12 M NP and double-stranded DNA was subsequently eluted with five 1-ml washes of 0.4 M NP. The respective washes were pooled and the amount of radioactivity in the pooled samples was measured by counting a fraction with a liquid scintillation counter. Between 90 and 103% of the tracer DNA was recovered in all cases. The data were analyzed using a computer program which derives second-order components from reassociation kinetics data using a least squares approach (Pearson et al. 1977).

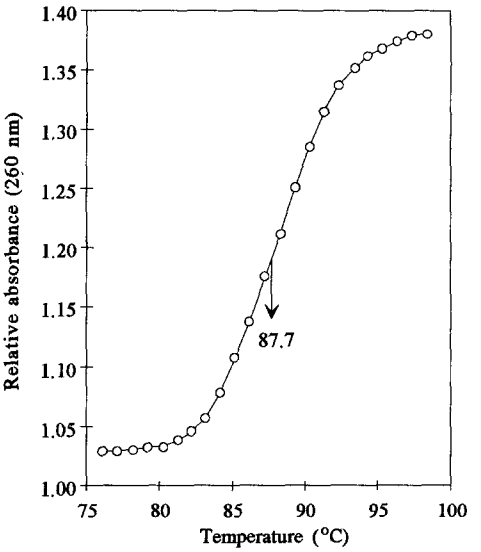

Fig. 1. Thermal denaturation profile of *P. graminis* DNA. Relative absorbance is the ratio of the absorbance at the elevated temperatures to the base-level absorbance after correction for thermal expansion (Mandel and Marmur 1986). The midpoint of the transition (T_m) is indicated with an *arrow*

Determination of the total DNA content of urediniospores. Urediniospores were disrupted in the MSK Braun homogenizer as described above except that after 2 min at 2000 rpm, tracer DNA was added and the sample was then homogenized for an additional 2 min. The tracer DNA consisted of *E. coli* DNA labeled by replacement synthesis (Sambrook et al. 1989). Unincorporated nucleotides were removed by repeated washes in a micro-concentrator and the absence of free nucleotides was confirmed by thin-layer chromatography (Johnson and Walseth 1978). The DNA in the spore homogenate was concentrated by CsCl-ethidium bromide equilibrium gradient centrifugation. The quantities of tracer and total DNA in the final extract were determined by liquid scintillation counting and the diphenylamine assay (Burton 1956), respectively. The numbers of urediniospores for calculating average mass were determined using a hemocytometer (Rowell 1984).

Results

DNA characteristics and base composition

For these studies, 3.0 mg of highly purified *P. graminis* DNA were prepared from 48 g of urediniospores. This

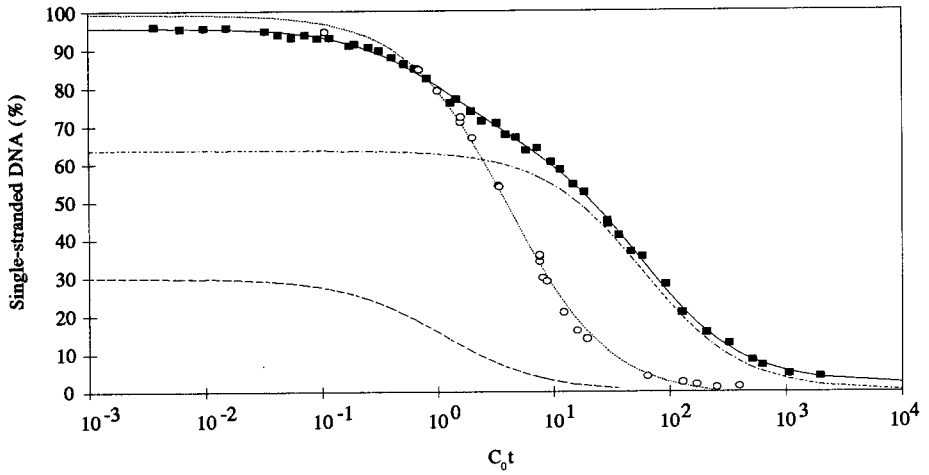

Fig. 2. The reassociation kinetics, as measured by hydroxyapatite chromatography, of *P. graminis* DNA and *E. coli* DNA. The curves labeled 'total' represent the best least squares solutions of the reassociation data assuming second-order kinetics (Pearson et al. 1977). The curve derived for *P. graminis* has two components: a unique sequences component ('unique'), and a repetitive sequences component ('repetititive'). ■ *P. graminis*; ○ *E. coli*; —— *P. graminis* total; – – – – *P. graminis* repetitive; –·–·– *P. graminis* unique; ····· *E. coli* total

Table 1. Analysis of DNA reassociation of *P. graminis*

Component	Fraction of genome	Rate constant		Complexity (Mb)	Copy number
		Rate constant[a]	SD[b]		
Foldback	0.04	–	–	–	–
Repetitive	0.30	9.02×10^{-1}	8.35×10^{-2}	0.39	52
Single copy	0.64	1.74×10^{-2}	9.02×10^{-4}	42.5	1

[a] In liters · mole^{-1} · second^{-1}
[b] Standard deviation of rate constant in liters · mole^{-1} · second^{-1}

Table 2. Determination of the DNA content of *P. graminis* urediniospores

Experiment	Spores disrupted[a]	Total DNA (μg)[b]	DNA per spore (fg)
1	6.83×10^{8}	279	408
2	5.05×10^{8}	217	430
3	7.63×10^{8}	357	468

[a] The homogenate was assayed for unbroken spores following spore disruption. The estimated total number of unbroken spores was subtracted from the total introduced into the flask. The percentage of the spores that remained unbroken was typically 5%
[b] The total DNA is the calculated total DNA of the disrupted spores. This was determined by first quantifying the radioactivity and the DNA in 50 ml of the final extract. The fraction of the introduced radioactivity present in the 50-ml aliquot was calculated and the total DNA obtained by dividing the DNA content of the aliquot by the fraction recovered in the aliquot

DNA had the following characteristics: average fragment length of 6 kb as determined by gel electrophoresis, an absorbance ratio of 1.86 (260:280 nm), hyperchromicity of 27%, and a melting temperature (T_m) of 87.7 °C. The thermal denaturation profile of *P. graminis* DNA is presented in Fig. 1. From the melting temperature, an estimate of the base composition was calculated using the equation $T_m = 16.6 \times \log C_s + 41 \times \varkappa_{gc} + 81.5$, where C_s is the total cation concentration and $\varkappa_{gc}$ is the mole fraction of guanosine plus cytidine (G + C) (Cantor and Schimmel 1980). With a T_m value of 87.7 °C, the G + C base composition of *P. graminis* DNA was determined to be 45.3%.

Reassociation kinetics

The reassociation kinetics of *P. graminis* and *E. coli* DNA are presented in Fig. 2. The curves identify the equations which best represent the data when evaluated for second-order kinetics components using a least squares approach (Pearson et al. 1977). To determine the number of components which best describe the *P. graminis* data, models with one, two, and three second-order components were tested. Only the two-component model yielded a reasonable solution. A very low root mean square error (0.0085) was obtained, indicating that the model accurately represents the data. The characteristics of these components based on the reassociation rate constant determined for the *E. coli* standard ($K = 0.249\ M^{-1}\ s^{-1}$) are presented in Table 1. The largest component, representing the single-copy sequences, contained 64% of the DNA and had a complexity of 42.5 Mb. Thirty percent of the DNA consisted of repetitive sequences, with a total complexity of 0.39 Mb, which are repeated an average of 52 times per haploid genome. Four percent of the DNA contained foldback sequences that reassociated prior to the first time point and 4.4% of the sequences did not reassociate. From the complexity and genome fraction of the single-copy sequences, the haploid genome size was estimated to be 67 Mb.

Quantification of urediniospore DNA content

To overcome the thick urediniospore wall, we employed the MSK Braun homogenizer for spore disruption. To

determine the percent recovery of DNA following extraction, tracer DNA was added to the spore extract midway through the disruption process. The DNA was then concentrated on CsCl gradients and the amount of tracer DNA and total DNA were determined. Typically, 50% of the tracer was recovered in the final DNA fraction. In three separate experiments the DNA content per urediniospore ranged from 408 to 468 fg (Table 2) with an average of 435 fg. This value is approximately three times the expected value of 147 fg, based on a genome size of 67 Mb and dikaryotic spores with both nuclei in the G_1 phase of the cell cycle.

Discussion

The haploid genome size of *P. graminis* was estimated to be 67 Mb based on an analysis of DNA reassociation kinetics. Previously, we had estimated the genome size to be 57 Mb (Backlund 1991; Backlund and Szabo 1991) based on an *E. coli* genome size of 4.2 Mb (Cairns 1963). The present estimate of 67 Mb is based on an *E. coli* genome size of 4.7 Mb, which was derived from a physical map of *E. coli* isolate W3110 (Kohara et al. 1987). We determined the rate constant of *E. coli* isolate W3110 to be $K = 0.429\ M^{-1}\ s^{-1}$ under our conditions.

At 67 Mb, the *P. graminis* genome is slightly larger than the estimated 45–50 Mb for *P. sorghi* (Anderson 1991; P. Anderson, personal communication), the only other rust fungus which has been characterized by reassociation kinetics. These findings are similar to those obtained from the reassociation kinetics of several other plant pathogens, including *P. megasperma* f. sp. *glycinea* (62 Mb, Mao and Tyler 1990) and *B. lactucea* (50 Mb, Francis et al. 1990).

Recently, the relative genome sizes of a number of rust fungi, including five species of *Puccinia*, two species of *Uromyces*, and one of *Melamspora*, were determined cytologically by measuring the fluorescence of propidium iodide-stained nuclei in basidiospores (Eilam et al. 1992). The genomes of *P. graminis*, *P. sorghi* and *P. coronata* were similar in size while the remainder were larger, ranging from 1.6 to 6.9 times that of *P. graminis*. Of the three that were similar in size, *P. sorghi* was found to be the smallest, being 0.8 times that of *P. graminis*, a finding consistent with the results obtained from the reassociation kinetics of the two species (45–50 Mb for *P. sorghi*, Anderson 1991; 67 Mb for *P. graminis*, the present study).

The analysis of the reassociation kinetics of *P. graminis* DNA also revealed that the repeated DNA sequences fell into a single class. These sequences, which comprise 30% of the total, have a complexity of 3.9×10^5 bp and are repeated an average of 52 times. In terms of the overall amount of repeated DNA sequences, *P. graminis* is similar to the fungi *Achlya bisexualis* (Hudspeth et al. 1977) and *Dictyostelium discoideum* (Firtel and Bonner 1972). In contrast, some 60% of the *B. lactucae* genome is comprised of repeated DNA sequences which can be divided into two classes: a middle repetitive class with an average repeat of 40 copies per genome, and a highly repetitive class with an average repeat of 1 200 copies per genome (Francis et al. 1990).

Although the nature and function of most repeated DNA sequences is not known, one sequence which can be expected to contribute to the repetitive fraction is the ribosomal repeat. We estimate that the *P. graminis* ribosomal repeat accounts for approximately 5% of the repetitive fraction based on preliminary estimates that this sequence is 9.5 kb long with 110 copies per genome (unpublished data). This is similar to *Aspergillus nidulans*, which is thought to have an 11-kb ribosomal repeat with 63 copies per haploid genome (Timberlake 1978). The ribosomal repeat appears to represent a much larger proportion of the repetitive fraction in the zygomycete *Absidia glauca*, however. Wöstemeyer and Burmester (1986) found that 13% of the *A. glauca* genome consists of ribosomal sequences which, given a median ribosomal repeat length of 11 kb, would be equivalent to 425 copies.

Whereas most studies of DNA reassociation kinetics have been conducted using only nuclear DNA, the DNA extraction procedure we employed yielded total DNA including both nuclear and mitochondrial DNA. In a study of *P. graminis* f. sp. *avenae* (closely related to the *forma specialis* we used, *P. graminis* f. sp. *tritici*) the mitochondrial genome was shown to be 80.2 kb in size with an average of 21 copies per urediniospore germling (Sock et al. 1991; J. Sock, personal communication). Assuming that the mitochondrial genome is similarly represented in *P. graminis* f. sp. *tritici*, mitochondrial sequences would account for only 1% of the repetitive sequences if the nuclei are in the G_1 phase of the cell cycle, or 2% if they are in G_2 as seems likely (see following paragraph). Taken together, the mitochondrial and ribosomal sequences appear to account for no more than about 7% of the repetitive fraction.

Given a genome size of 67 Mb per genome and two genomes per dikaryotic urediniospore, a single urediniospore should contain 147 fg of DNA. Chemical analysis of the DNA content of urediniospores, however, showed that the spores contained approximately 435 fg or three-times as much DNA as expected. These results suggest that the spore may be in the G_2 phase of the cell cycle. This interpretation is consistent with results obtained from urediniospores of *Uromyces appendiculatus* which indicate that nuclei are in G_2 during germination, and undergo a mitotic division prior to DNA synthesis and differentiation (Kwon and Hoch 1991).

The accuracy of our estimate of the DNA content of urediniospores depends on the assumption that the partitioning of the radioactively-labeled tracer DNA accurately reflects that of the endogenous DNA. It is possible that the two DNAs would not partition equivalently since the tracer was naked DNA whereas the endogenous DNA would have been associated with proteins. The disruption process is quite harsh, however: the average fragment length of endogenous DNA immediately following spore disruption is only 6 kb, indicating that the nuclear DNA-protein complex was extensively disrupted with the result that much of the endogenous DNA may have been in a naked state. Given this level of disruption, it is rea-

sonable to assume that the two DNAs partitioned similarly.

Another aspect of the structure of rust genomes that has been illuminated recently is the chromosome complement. Ultrastructural studies have shown that *P. graminis* (Boehm et al. 1992) and *M. lini* (Boehm and Bushnell 1992) both possess 18 chromosomes while *P. coronata* has 17 (Boehm 1992). These n values, larger than had been indicated earlier by light microscopy, are similar to those of other plant pathogens being developed as model systems; e.g., *Cochliobolus heterostrophus* with an n value of 16 (Tzeng et al. 1992) and *Ustilago maydis* with an n value of 20 (Budde and Leong 1990).

Wheat stem rust, the most extensively studied of all the cereal rusts, is an attractive candidate for investigating the molecular biology of host-parasite interactions in ceral rust diseases given its economic importance, its extensive physiological characterization, and the elaborate and well detailed genetic interactions between the pathogen and its host. The results presented here, along with those of several recently published studies pertaining to rust genome size and karyotype, suggest that *P. graminis* is a promising candidate for further study, and is comparable in genome organization to other plant pathogens currently being analyzed at the molecular level.

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